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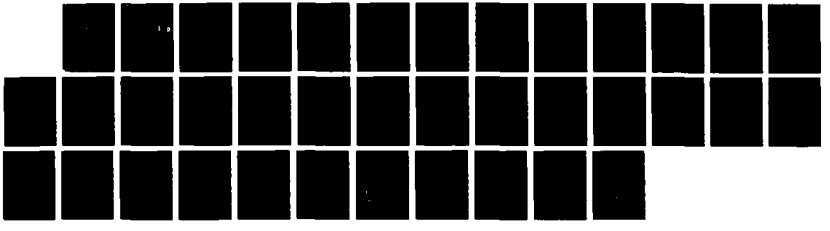

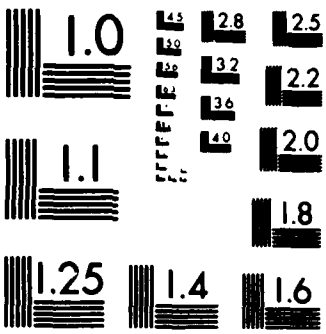

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STUDIES OF ALTERED RESPONSE TO INFECTION INDUCED BY THERMAL INJURY

ANNUAL PROGRESS REPORT

Carol L. Miller, Ph.D.

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This contract period has been particularly productive. We have developed the MØ assay, and have accumulated data indicating that this new assay is monitoring an important burn altered MØ function. We are improving and refining the PGE_2 assay while gathering important new indications of the inimical effects of post-burn elevation of MØ PGE_2 on immune function. We also now have the capacity to identify human T_s subsets using the fluorescence cell sorter and expect to expand this capacity in the coming year. Most important, we are now beginning to examine various prophylactic treatments for their efficacy in reversing a number of burn mediated immune defects. We expect even more interesting data and several publications to result from experiments now in progress.

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FOREWORD

For the protection of human subjects, the investigator has adhered to policies of applicable Federal Law 45CFR46.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION

The high incidence of fatal septicemia associated with severe thermal injury is believed to result from loss of immunocompetence. This laboratory has been able to identify those burn patients who are at greatest risk for developing fatal sepsis by detecting the loss of certain immune functions by cells of these patients. Besides designing assays to monitor the critical burn-induced immunodefects, our experiments have focused on those mechanisms which, when triggered by severe thermal injury, can lead to cellular immune aberrations. Direct burn-induced immune dysfunction can result from aberrations in any of the three general types of leukocytes which cooperatively mediate the generation of immune function. These three leukocyte subpopulations are the antigen specific bone marrow derived (B) cell, the antigen specific thymus-derived (T) cell, and a third extremely heterogeneous population of leukocytes - the monocyte or macrophage (MØ).

During this contract year our focal points have been studying burn-induced alterations in monocyte (MØ) function, characterizing the spectrum of burn-induced regulatory cells and examining possible therapeutic modalities designed to reverse or diminish these MØ and T_s dysfunctions. We have initiated two new MØ assays (measurement of endogenous pyrogen (EP) and complement components) as well as refining the PGE_2 assay which was introduced last year. Our working hypothesis has been that critical alterations in MØ function occur very early in the post-burn period. These early changes in MØ activities unbalance the immune network away from immunocompetence and toward excessive regulation and hypoimmunity.

The monocyte population appears to be divided into facilitory (fac) and inhibitory (inh) subsets just as the T lymphocyte population is segregated into helper (T_h) and suppressor (T_s) cells. A complex reciprocal interaction occurs between fac MØ and T_h cells. Recent data indicates that a similar reciprocal interchange occurs between inh MØ and T_s cells.

If thermal injury can interrupt and/or alter certain pivotal MØ functions, both PMN function and immune function will be disrupted. This laboratory has been

monitoring a number of MØ functions which are critically involved in host defense. The monocyte functions, as well as the immune functions of thermally injured patients, are assessed every 3 days from their admission to their release or demise. Alterations in these MØ activities are determined by comparison to the patients' own initial MØ activity level and to the established "normal" level. The patients' monocytes are routinely monitored for their production of plasminogen activator (PA), tissue procoagulant factor (TF) and lysozyme (Ly). Recently, we have also been routinely monitoring all severe burn patients' MØ for PGE_2 production. In addition, we have now begun to monitor selected burn patients' MØ (those identified as at high risk by mitogen assays) for their production of endogenous pyrogen (EP) and complement component 2 (C2). Both of these MØ products are crucial to the inflammatory response. There is also evidence that these two products (EP and C) may have major effects on the functions of specific immune cells. This laboratory has developed several more sophisticated means of identifying burn-induced T_s and inh MO. We are utilizing both Fluorescence Activated Cell Sorting (FACS) and specific depletion with monoclonal anti-T or MØ antibodies to delineate the role of T_s and MØ subset in immunoincompetent burn patient syndrome. In addition, we have begun developing experiments designed to test various drug therapies for their efficacy in restoring what appear to be critical MØ and/or T_s aberrations. This contract period has seen considerable advancement not only in monitoring burn patients' cellular defects but also in defining the critical mechanisms of burn-induced immunodefects and in examining several therapeutic modalities. The work of this year has been reported in three papers and four abstracts.

METHODS

Human Studies

Normal volunteers (medical staff) are utilized as donors of control human leukocytes. Consenting asplenic trauma patients are assigned an ISS score. Data from these patients' assays are assessed by comparing ISS scores of 9-25, 25-35 and scores $>$ 35. Similarly, thermally injured patients are selected according to the following criteria: 18-34 years of age $>$ 40% 2° and 3° burn, 35-54 $>$ 30% 2° and 3°, 55-64 $>$ 10% 2° and 3°, and 65^{+} $>$ 5% 2° and 3° burn. A qualified physician collects the blood samples to assure appropriate safety precautions are always observed. Minors, prisoners, pregnant women and the mentally handicapped are excluded as donors.

All patients assayed are first drawn on admission and then drawn again every 3 days until release or demise. Approximately 20 ml of blood is collected on each assay day. Mononuclear cells are isolated from the peripheral blood (PB) by Ficoll-Hypaque gradient centrifugation. The isolated cells are simultaneously tested in the PHA, PA, TF, mitogen, PGE_2, C, EP and lysozyme assays. Only selected patients (those judged on clinical evidence to be at high risk) are assessed for T_s activity. Patient mononuclear populations can be further depleted of T cells, monocytes and/or B cells. The isolation of T cell subsets can be accomplished in one of two ways. In one set of experiments we are utilizing commercially available monoclonal antisera (OKT4 = helper/inducer; OKT8 = suppressor/cytotoxic) and the Fluorescence Activated Cell Sorter (FACS). We have developed a collaborative arrangement with Dr. Marvin Garovoy and sort T subsets on a Becton-Dickinson Fluorescence Activated Cell Sorter. FACS sorting involves a positive selection of suppressor cell subpopulations by means of the FACS and fluoresceinated specific anti-T cell monoclonal antibodies such as OKT8 and OKT4 (Ortho Pharmaceutical). The E-rosette positive T lymphocyte enriched cell population (0.25 ml of 1.5×10^7/ml) is incubated with 0.25 ml of 1:100 dilution of fluoresceinated antibody for 45 minutes in the dark on ice. The cells are washed three times and diluted to a final concentration of 1×10^6/ml for sorting. Both patient cells and Con A generated cells are sorted and assayed for their effect on MØ production of plasminogen activator or T helper cell activity. Additionally, the $OKT8^+$ cells are collected from the FACS sorter. Purported suppressor cell subsets are harvested, washed, and resuspended at either a concentration of 5×10^6/ml or a concentration equivalent to the positively selected FACS cells. Cells are added to isolated MØ. We are also further segregating the $OKT4^+$ suppressor-inducer utilizing Ortho monoclonal OKT17.

We also employ specific anti-T cell antibody to deplete various T subpopulations by treating the cells with the specific monoclonal antibody and complement. Again, these cells are assayed for their effect on MØ production of plasminogen activator. In this manner we can positively select for a given cell population using the FACS or negatively select for a cell population by lysing it with antibody and complement.

We isolate MØ by selective adherence of the mononuclear cell populations to flasks by the Ackerman and Douglas rapid adherence technique. These MØ populations can be further isolated by positive selection with fluoresceinated antibody (OKM5) and FACS sorting or negative selection by Sephadex G-10 passage and/or antibody and

complement treatment. The B cells can be removed by nylon wool filtrations of the cell population.

We monitor the ability of patient and normal mononuclear cell populations to respond to phytohemagglutinin (PHA). This non-specific mitogen response requires the cooperative interaction of monocytes and T cells. The isolated monocytes are routinely examined for the production of PA, their level of tissue procoagulant factor (TF), their generation of prostaglandin E_2 (PGE_2), Endogenous Pyrogen (EP) and their synthesis of lysozyme. MØ from selected patients are also assayed for their production of C2. In the PA assay, patients' and normal controls isolated MØ are place into ^{125}I-fibrin plates and cultured 18 hours either in the presence of acid treated fetal bovine sera (AT-FBS) or soybean trypsin inhibitor (SBI), an inhibitor of plasmin. After all the PA is released in these cultures, the cells are washed and fresh AT-FBS media or SBI media are added for an additional 24 hour incubation period. The amount of fibrinolysis initiated during this second incubation period is then measured. Monocyte numbers are adjusted to produce approximately 25-35 fibrinolytic units for normal individuals (4×10^5 isolated MØ). Simultaneous to our assessment of patients' monocyte's PA synthesis, we also assay their production of TF and lysozyme. TF production is measured using the Rickle's assay and lysozyme production is measured during the Schill and Schumacher Lysozyme Plate test.

Samples used in the PGE_2 assay are obtained from the MØ supernates of burn and trauma patients. Normal MØ supernates are run simultaneously for each assay as a control. Briefly, mononuclear cells are separated from peripheral blood by Ficoll-Hypaque centrifugation. The cells are incubated on flasks (8×10^6 cells/ml) for 1½ hours to select for the monocyte population. Fresh media is put on the flask and the cells are incubated overnight. The media and the adhered cells are removed from the flask and cell counts are taken. The media is aliquoted into 2 ml samples which are stored at -85° C until extraction. Currently, we are using a commercially prepared solid-phase extraction column (Sep-pak C18, Waters Assoc., Milford, Mass.) and then quantitatively assaying the samples with an ^{125}I-PGE_2 radioimmunoassay kit (New England Nuclear, Boston, Mass.). A standard curve is prepared utilizing a range of 25 pg/.1ml to 0.25 pg/.1ml. 100 ul of the standards and the samples are aliquoted into the corresponding polypropylene tubes. 100 ul of PGE_2 [I^{125}] tracer is added. 100 ul of rabbit PGE_2 antibody is added and the tubes are vortexed. Tubes are incubated overnight at 2-8° C. 16-24 hours later tubes are put on ice and 1 ml of

cold precipitating reagent is added. Tubes are vortexed and incubated on ice for 20-30 minutes. After incubation, tubes are centrifuged at 2,500 rpm at 28° C for 30 minutes. Supernates are decanted and the residue is counted on a gamma counter for 1 minute count time. Counts are converted to percentage bound and are then compared to the standard curve for PGE_2 concentrations. We are currently in the process of initiating a new method for the detection of MØ production of PGE_2 in patient supernates. We are utilizing the classic radioimmunoassay for PGE_2 as described by Wahl. This assay system utilizes a new and monospecific antisera (Boehringer-Mannheim) for PGE_2 and a $[^3H]$-PGE_2 (Amersham) tracer is used rather than $[I^{125}]$-PGE_2. A standard curve covering the range of 12-3,000 pg PGE_2 is prepared.

100 ul of the standards and the samples are aliquoted into the corresponding tubes. 50 ul of $[^3H$-$PGE_2]$ is added to all tubes and mixed by vortexing. 50 ul of anti-PGE_2 antisera are added to all the tubes and vortexed. Tubes are then incubated at 37° C for 1-2 hours. 100 ul of normal rabbit serum and 100 ul of goat anti-rabbit serum are added to the tubes and vortexed. Tubes are then incubated at 4° C for at least 18 hours. After incubation, tubes are centrifuged at 1,900xg for 30 minutes at 4° C. Supernates from tubes are decanted and discarded. 1 ml of Buffer (50 mM Tris, pH 9.0) is added to each tube and the tubes are then vortexed to solubilize precipitate. The solubilized precipitate is placed in scintillation vials with 10 ml of scintillation fluid. Samples are counted for 1 minute on a scintillation counter. Counts are converted to percentage bound and are then compared to the standard curve.

The cell free supernates collected from MØ during isolation of the Ackerman Douglas flasks can be assessed for EP as well as for PGE_2. These supernates are assessed by using a minor modification of the method previously described by Bodel and Miller. Briefly, 12 week old male Balb/C mice are prewarmed to a slightly higher than basal temperature of 38-40° C. After 1 hour, they are removed every 10 minutes for rectal temperature readings, taken by means of a thermistor probe (Yellow Springs Instrument Co., Yellow Springs, Ohio), inserted to a distance of 2 cm for 1 minute. After 50 minutes, stable baseline temperatures are achieved, at levels between 36.5° C and 38° C. These mice were then injected intravenously with 0.3 ml of test patients' supernates. Temperatures of the mice are monitored every 10 minutes thereafter for 50 minutes. Individual mice receive no more than three injections over a three week period before they are sacrificed.

When a patients' MØ are to be examined in the complement assay, additional Ackerman-Douglas flasks are prepared as described above. The flasks are separated

into different groups; unstimulated cells (used as a control) and cells stimulated with Fc fragments (50 mg/ml), PHA (1 mg/ml) or PPD (10 mg/ml). The cells are cultured for two days or four days and then assessed in the complement assay. The synthesis of the C2 complement component by the monocyte is measured by the hemolytic plaque assay as developed by Colten. Briefly, the cell suspension from the cultured flasks (at 4×10^6/ml) are mixed with stable cellular intermediates EAC_{14} (Cordis Lab) at 10^9/ml, and with 0.5% agarose then left at 47° C in a water bath. This mixture is poured on a coated slide. These slides are incubated for about one hour at 37° C. Hemolytic plaques appear after 40 minutes incubation at 37° C with 1/40 EDTA treated rat complement.

We are also employing an improved hemolytic plaque assay method described by Alpert (1). This is a thin monolayer method that should be able to increase the sensitivity in detecting the synthesis of C2 and C4 components by single cells.

Human blood mononuclear cells are isolated by centrifugation on Ficoll-Hypaque. Adherent purified monocyte monolayers are prepared by layering cell suspensions onto glass coverslips. The coverslips (with adherent cells on the upward surface) are placed on a thin layer of solidified agarose in a small petri dish. The indicator cells EAC_{14} for C2 PFC (plaque forming cell) or EAC_1 (for C4 PFC) are added to an agarose solution, vortexed and poured over the coverslips in the petri dish. The dishes are then incubated and the C2 PFC are revealed by adding the EDTA-treated rat complement. The C4 PFC are revealed by adding guinea pig C2 followed by EDTA-treated rat complement.

Utilizing our present assay system, from one 20 ml blood sample, we can simultaneously measure PHA, MØ PA generation, MØ PGE_2 and EP synthesis, MØ lysozyme and TF production and MØ complement synthesis. To detect suppression we need to draw additional blood samples. Human mononuclear cells are separated into T, B or MØ subpopulations and the interaction between these subpopulations is evaluated. The effect of suppressor T cells or of inhibitory MØ is assessed by mixing purified autologous cell populations and assessing the affect of one cell type (i.e., suppressor T cell) on another cell populations' function.

A new assay for measuring burn-induced suppression has been developed by this laboratory. This assay assesses the ability of burn patients' E-rosette positive T cells and/or MØ to suppress PA production by a normal individuals' MØ. The burn-induced suppressive cells are incubated 24 hours with isolated normal controls' MØ, the normal MØ are re-isolated, adjusted to 4×10^5/well and assayed in our PA

system as described above. We compare the PA production of MØ incubated with allogeneic burn patients' cells to their PA production after exposure to allogeneic normal individuals' cells.

We have detected burn-induced aberrations in the immune regulation functions of patients' cells using a modification of the classical mixed lymphocyte response (MLR). In our MLR system, a highly responsive combination of cells from two normal individuals is cultured in a "one way" MLR. In this assay, one group of the normal's cells are pretreated with mitomycin C (MC) to prevent their division. Consequently, this "one way" MLR assay measures the ability of one group's normal cells (Responder=R) to proliferate in response to the foreign histocompability antigens on another normal's cells (Stimulator=S). We compare the effect of adding either burn patient cells or MC treated responder cells on the amount of proliferation measured in the MLR cultures.

Data Calculation and Statistical Analysis

The data presented for patient and normal's PA production is always from the second incubation interval. All supernates CPM's of ^{125}I-fibrin are corrected for media and non-specific radioactivity release by subtraction of CPM's obtained from the no-cell control. The CPM's of ^{125}I-fibrin in the supernates from the lines containing cells in 100 ug SBI are subtracted from the CPM's of lines containing the cells in AT-FBS. This corrects for any ^{125}I-fibrin lysed by any non-plasmin mediated mechanisms. This corrected AT-FBS CPM is then divided by the total ^{125}I-fibrin CPM's present to derive the percent specific plasmin mediated lysis. This value is computed for patient cells collected every four days post-injury. The mean and standard deviation of PA production by MØ from 43 normal individuals tested repeatedly was 25 $\pm$ 8.4. The patient data were calculated by comparing the PA response at various days post-injury to both the normal values (25 $\pm$ 8.4), and their own initial (day 1) values. A Student's t-test was used to determine significant differences. The TF activity of sonicates from 10^5 MØ was calculated in thromboplastin equivalent units by comparison of the shortened thromboplastin time to a control brain thromboplastin standard curve.

In addition, we are analyzing the PGE_2 as follows: Counts are averaged for each set of duplicate samples. Average "net" counts are calculated for all standards and samples by subtracting the average "blank" count from each value. The normalized percent bound (%B/Bo) is then found for each standard and sample as follows: % B/Bo = (net counts of standard or sample/net count of 100% B/Bo standard) x 100. The % B/Bo standard versus the corresponding picograms of PGE_2 added to each standard is

plotted using semi-logarithmic graph paper. Sample values are interpolated from this standard curve to yield Pg of PGE_2. This must then be adjusted to $Pg/10^6$ cells in the following manner: (Pg of PGE_2/100 ul) x (1000 ul/1 ml) x (#ml Patient Sample/cell count MØ) x (dil factor).

The PGE_2 assay requires extensive data processing. We have written a computer program to handle these data. Unfortunately, the Hewlett-Packard computer we are sharing time on has become almost inaccessible. Not only do we use it for the PGE_2 data but also to process the PA, lysozyme and C data. Presently, we face delays on data calculation of up to one week. As requested under separate cover, we would like to purchase a microcomputer system for this laboratory.

Human peripheral blood mononuclear cell populations differ from individual to individual in their percentage of MØ, T and B cells and their degree of immune reactivity. It has been suggested that human immune functions are controlled by immune response genes analogous to those described in animal systems. Consequently, the "normal" levels of MØ PA production, mitogen responsiveness, MØ TF generation, lysozyme production, and MØ PGE_2 activity vary for each patient and within the normal control groups. The baseline levels of each individual's MØ and T cell activities are not randomly distributed. Some individuals are low and some are high responders. This non-binominal distribution of the MØ and T cell parameters necessitates the use of non-parametric statistics when analyzing patients' data. We utilize the Wilcoxon test for evaluating the statistical significance alterations in patients' mitogen, PA, and TF assays. We utilize Spearman's correlation coefficient for determining the degree of interdependence between the various MØ and T cell parameters.

Guinea Pig Experiments

70 strain 13 inbred guinea pig of both sexes 300-400 grams in weight were used in these experiments. The monocytes' dependency of the guinea pig immune response is much greater than that seen with the murine system. An *in vitro* prime is therefore necessary in order to measure an *in vitro* immune response. The guinea pig *in vitro* secondary response (like the human system) is much more subject to disruption than the mouse. Consequently, the guinea pig is a more comparable model for evaluating immune disfunctions in thermally injured patients.

A primary challenge is administered by subcutaneous injection of 0.4 ml complete Freund's Adjuvant (Gibco) and 2% sheep red blood cells (Gibco) emulsion in the footpad. On day six after the primary challenge, some of the guinea pigs are anesthetized with 60 mg/kg body weight Ketamine and receive a 20-30% total body

surface area third degree scald burn (90° C 30 sec). The sham injured guinea pigs were anesthetized but not burned.

Three injections of TP5 were given. At four to six hours, 24 hours, and 48 hours post-burn one group of the guinea pigs received an IV injection of 1 mg/kg TP5 (Thymopentin, Ortho Pharmaceutical Corporation). In later experiments, the TP5 dosage was increased to 3 mg/kg body weight. Finally, 3 mg/kg TP5 was injected IV in combination with 1.5 mg/kg Indomethacin (Sigma) administered intraperitoneal. A second group of burned guinea pigs along with the sham injured guinea pigs received equivalent IV injections of saline.

At day four post-burn, the guinea pigs were sacrificed by CO_2 asphyxiation and the spleens sterilely removed. The in vitro generation of antibody forming cells has been previously described.

The number of antibody forming cells (AFC) to sheep red blood cells (SRBC) is assayed using the slide modification of hemolytic plaque assay. Plaques are visually counted.

Spleen cells were maintained five days in a modified Mishell-Dutton culture system. The Mishell-Dutton system was modified as follows: Six-well tissue culture plates were used to culture 1.2×10^7 cells in 1 ml Iscoves liquid media supplemented with a final concentration of 1% Penicillin/Streptomycin, 1% L-Glutamine, 0.5% L-Asparigine (all Irvine Scientific), 1% Garamycin (Scherring), 1% Trypticase Soybroth, 5×10^{-5}M 2-Mercapto-ethanol and 4% heat inactivated Rabbit Serum (Kappa Scientific). 50 ml of one percent SRBC were used as immunogens in test wells, each test well had a control well with SRBC.

The in vitro generation of AFC is assayed using the slide modification of the Hemolytic Plaque assay. Leukocyte recovery from cultures is determined by counting a sample of the harvested, cultured cells on a Coulter Counter (Model ZH). The number of AFC are calculated for each pool of duplicate background plaques and expressed as AFC/10^6 recovered spleen cells Allogeneic conditioned media is produced as described.

RESULTS AND DISCUSSION

In this time period, we have studied an additional 9 severely burned individuals. These 9 individuals ranged in age from 29-83. All these individuals suffered $>$ 30% 3°. They were included in the study using the age vs total body surface burn criteria described under Methods. Of these 9 patients, 5 succumbed to overwhelming sepsis. We retrospectively divide the patients into three categories based on

alterations in their mitogen responses. Group I patients exhibit less than a 33% alteration in their mitogen response and have an uneventful clinical course. Group II patients exhibit a hyper-mitogen response usually in response to an infectious episode. This mitogen hyperimmunity is typical of a normal immune system dealing with an infectious challenge. The Group II patients have a clinical course characterized by an infectious episode which they overcome. The Group III patients show a profound and early depression of their mitogen response. Their clinical course is punctuated with repeated septic episodes from which the patient often succumbs. As illustrated in Table I, depression of the mitogen response is accompanied by depressed facilitory (fac) MØ function as indicated by depression in MØ production of Plasminogen Activator (PA). This decrease in fac MØ function is not accompanied by cessation of all MØ functions. Lysozyme production is either unchanged or increased in these patients. These data indicate that MØ functions are selectively effected. As can be seen in Table I, all of the Group III burn patients show a decrease in PA production and this depression appears earlier than mitogen hyporesponsiveness (2-4 days post-injury) and persists even when the mitogen responses have returned to normal. These data are consistent with our hypothesis that severe thermal injury mediates earlier changes in critical MØ functions. If these changes in pivotal MØ functions are extensive enough, not only immune function, but also other host defense systems such as neutrophil chemotaxis and phagocytosis are critically depressed.

Another experimental design we are using tests our hypothesis that early MØ alterations are pivotal in overall depression of host defense. This experimental design involves monitoring MØ PGE_2 production. Excessive PGE_2 levels can directly suppress MØ function, lymphocyte function, and PMN maturation while increasing T_s generation. Consequently, an increase in PGE_2 levels could be a primary trigger of many of the alterations seen post-burn. If elevated PGE_2 levels are the major contributors to post-burn immunodepression, then specific treatments (such as indomethacin which is antagonistic to PGE_2 synthesis) might reverse some or all of the post-burn immunodepression. Experiments examining indomethacin in an animal model are described in a later section. In our current patient assays, we have attempted to establish that an increase in MØ PGE_2 does occur in severe thermal injury and that this increase in MØ PGE_2 production correlates to clinical outcome. Table 2 illustrates data on PGE_2 levels in some of the patients studied during the past year. As can be seen in Table 2, only patients who later developed septic complications showed elevated PGE_2 levels at 1-4 days post injury (Group III

TABLE 1

CHANGES IN MITOGEN RESPONSE AND FACILITORY

MØ FUNCTION IN BURN PATIENTS

Patient	Maximum % PA Suppression	Maximum % PHA Variation	% Burn	Outcome
		GROUP I		
KO	-32%	+42%	44%	No complications released
		GROUP II		
MA	-22%	+286%	55%	Staph infection recovered
HE	-46%	+405%	35%	Pseudonomas infection recovered
		GROUP III		
HO	-88%	-87%	38%	Serratia, staph, candida, pseudomonas recurring infection finally recovered
MA	-69%	-90%	45%	Pseud septicemia expired
WE	-73%	-69%	36%	Staph, pseud, candida sepsis expired
KE	-77%	-83%	71%	Staph, pseud sepsis expired
RA	-69%	-70%	50%	Pseud septicemia expired
SY	-70%	-88%	50%	Serratia, staph sepsis expired

T A B L E 2

Correlation of PGE_2 with Massive Increase of PGE_2 at 1-4 Days Post-Burn

Patient	Max PGE_2 1-4 Days	Max PGE_2	Outcome
Group I			
AR	+200	+500	No complication released
PH	+4,113	+10,000	No complication released
Group II			
EL	+725	+11,566	Staph infection recovered
RI	+2,254	+34,503	Staph infection recovered
ZY	+1,404	+22,871	Pseudomonas infection recovered
Group III			
MO	+8,270	+8,270	Succumbed to staph sepsis
MC	+48,090	+48,090	Succumbed to pseudomonas sepsis

patients). Interestingly, Group II patients showed elevated PGE_2 levels late in their clinical course after their infectious episode. This late rise in PGE_2 may reflect a natural downturn mechanism to shut down the hyper-immune response (elevated PHA) that is characteristic of Group II patients. After the infectious challenge has been handled the normal regulatory mechanisms may decrease the responses to original levels in these patients. We have encountered some difficulty with the RIA kit we were initally using to measure PGE_2 levels in the MØ supernates. This kit requires an extensive extraction and then a conversion of PGE_2 to PGB. Both of these procedures have low efficiency and a highly variable product recovery. The consequences of these technique problems are that our accuracy in quantitating PGE_2 amounts was poor. When we ran different known quantities of PGE_2 through our assay system, we found that we could not detect amounts less than 15,000 pg and that we could not discriminate 50,000 pg from 100,000 pg. This means that when we detect 40,000 pg in patient samples using the ^3H-PGE_2 kit, the actual PGE_2 levels are much higher. This insensitivity is probably why patient PGE_2 production seemed to appear and disappear rather than progressively increase and decrease. We are now using a commercial RIA kit which detects PGE_2 directly (no conversion necessary) and requires only a column extraction rather than an ether lipid extraction. The columns for this extraction are commercially available and the whole procedure can now be completed on the same day.

In addition, we have obtained an even more specific anti-PGE_2 antibody and a purified H^3-PGE. We are also attempting to develop our own assay using these specific reagents using a modification of Wahl's assay (2). It is preferable to use a ^3H-PGE_2 label to ^{125}I-PGE_2. Tritium is easier to work with and has less danger of radioactive contamination. We expect to have this new improved assay functioning routinely in the next 2 months. At that point we should be able to detect subtle differences in MØ PGE_2 production post-burn.

Alterations in MØ PGE_2 production may also be affecting MØ production of Endogenous Pyrogen (EP). EP and Interleukin I (Il-I) appear to be two different activities of the same biological moiety. However, it also appears that there may be more than one molecular compound that has both EP and T cell mitogenic capacity (i.e. Il-I activity). One of these EP/Il-I moieties may be inducing T_s expansion and proliferation rather than T_h proliferation (3). This is especially relevant in light of the data we have recently been collecting monitoring the EP production by isolated patient MØ. As illustrated in Figure I, we have seen an increase in MØ EP

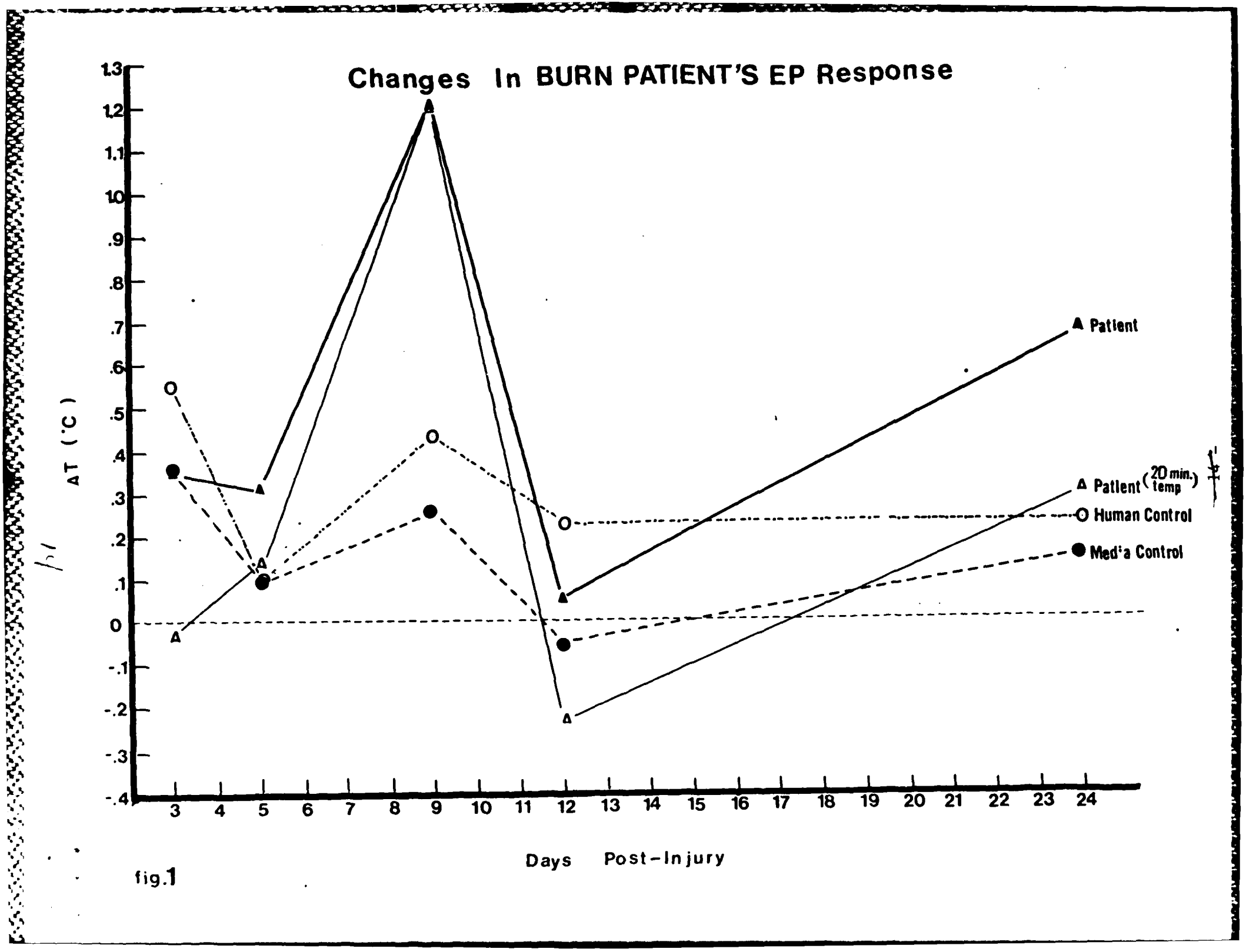

Changes In BURN PATIENT'S EP Response
ΔT (°C)
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
-.1
-.2
-.3
-.4
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Days Post-Injury
Patient
Patient (20 min. temp)
Human Control
Media Control

fig.1

LEGENDS

Fig. I. These data represent an 83 year old male with 37% burn. This Group III patient succumbed to fatal septicemia. Changes in burn patient's MØ response are measured by the ability of MØ supernatants to cause fever in mice. Data are presented as average temperature changes from baseline after injection of supernatants derived from 10^7 human monocyte incubated in 12% Fetal Calf Serum (FCS) media overnight.

—▲—▲— Patient = patient's injected supernatant (average peak temperature changes, ΔT, from baseline)

—▲—▲— Patient (20 min. temp.) = patient's injected supernatant (average peak temperature changes, ΔT, from baseline 20 minutes after injection)

—○—○— Human Control = normal human MØ supernatant is injected as a control

—●—●— Media Control = 12% Fetal Calf Serum (FCS) media incubated without cells is injected as a control

levels in mitogen hyporesponsive Group III patients whose MØ PA were severely depressed. In contrast, Group I patients showed no such higher EP response (Figure 2). This exciting and unexpected result has led us to consider several further experiments. We will be initiating an Il-I assay which detects human Il-I activity by its ability to cause murine thymocytes to proliferate. If the patients' EP supernates contain an Il-I which activates T_s, then the thymocytes exposed to this material should now suppress syngeneic murine splenocyte responses. If the increased EP we have detected in these patients is an indicator of increased MØ activation of T_s it would have major implications in the development of the immunocompromised burn patient syndrome. The alterations in patients MØ EP may indicate increased suppressor cell activation.

Concominant changes in MØ C synthesis would result in reduction of crucial phagocytic and opsonic activities. MØ synthesis of some of the C components (C4, C3, C2, C5, Factor B) controls their concentrations at the local injury site. Consequently, a decrease in MØ synthesis of various critical C components could lead to insufficient C levels at the injury site even though no decrease in serum complement levels was detected. We have monitored the level of C2 synthesized spontaneously and after <u>in vitro</u> stimulus with antibody fragments or lymphokines. Data in Tables 3, 4, 5, and 6 illustrate results from the complement experiments. We first established that the crystal fine fragment of antibody (Fc fragment) produced the best stimulation of C2 synthesis (Table 3). PHA induced T cell lymphokines would stimulate increased C synthesis, but this stimulation was more variable than that found with Fc fragments (Table 4). Data from the literature had suggested that PPD was a good stimulator of some monocyte functions. In our hands PPD failed to increase the levels of MØ C synthesis over that spontaneously seen in culture (Table 5). We also examined and compared two days of <u>in vitro</u> stimulus to 4 days of <u>in vitro</u> stimulus to ascertain where maximum C synthesis occurred. It appeared that most normal individuals showed tripling of C synthesizing MØ between unstimulated and stimulated cultures after only 2 days of incubation. After four days of culture the difference between stimulated and unstimulated MØ C synthesis was only twofold. However, the maximum absolute number of detectable plaques (i.e., C synthesizing MØ) was greater after 4 days of culture. Consequently, we have chosen to assay patient and normal responses after 4 days of culture to ensure that the maximum number of C synthesizing MØ is always detected. The assay is, therefore, weighed in the patients' favor and against detecting a defect. Even in this assay, however, it is quite apparent

Changes In BURN PATIENT'S EP Response
Human Control
Patient
Patient (20min. temp
Media Control
Days Post-Injury
ΔT (°C)
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
-.1
-.2
-.3
-.4
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

fig.2

LEGENDS

Fig. 2. This 35 year old male presented with a 44% burn. This Group I burn patient had an uneventful clinical course.

Patient = patient's injected supernatant (average peak temperature changes, ΔT, from baseline)

Patient (20 min. temp.) = patient's injected supernatant (average peak temperature changes, ΔT, from baseline 20 minutes after injection)

Human Control = normal human MØ supernatant is injected as a control

Media Control = 12% Fetal Calf Serum (FCS) media incubated without cells is injected as a control

TABLE 3

RANGE OF C SYNTHETIC RESPONSE BY NORMAL MØ AFTER STIMULUS WITH Fc

Plaques/10^6 MØ

NORMAL	2 DAYS OF CULTURE WITH STIMULUS		4 DAYS OF CULTURE WITH STIMULUS	
	NONE	F_c	NONE	F_c
a	10	22	10	18
b	7	32	10	30
c	9	16	10	20
d	2	10	10	27
e	2	4	15	20
f	2	10	3	12
g	6	16	5	28
h	0	9	9	19
i	8	18	12	17
j	6	15	13	20
k	10	20	10	15
l	—	—	12	20
m	—	—	10	30
$\bar{x}$ =	6 ± 3	16 ± 7	10 ± 3	21 ± 5

TABLE 4

RANGE OF C SYNTHETIC RESPONSE BY NORMAL MØ

AFTER STIMULATION WITH PHA INDUCED LYMPHOKINES

Plaques/10^6 MØ

	2 DAYS OF CULTURE WITH STIMULUS		4 DAYS OF CULTURE WITH STIMULUS	
NORMAL	NONE	PHA	NONE	PHA
a	2	2	4	4
b	7	15	10	17
c	2	15	10	20
d	6	17	13	12
e	6	16	5	24
f	0	5	9	15
g	–	–	10	12
h	–	–	10	19
i	8	12	–	–
j	–	–	3	15
k	–	–	12	16
l	–	–	12	15
$\bar{X}$ =	4 ± 3	12 ± 6	9 ± 3	15 ± 5

TABLE 5

FAILURE OF PPD TO STIMULATE NORMAL INDIVIDUAL'S MØ C PRODUCTION

Plaques/10^6 MØ

	2 DAYS OF CULTURE WITH STIMULUS		4 DAYS OF CULTURE WITH STIMULUS	
NORMAL	NONE	PPD	NONE	PPD
a	2	2	4	4
b	10	8	10	7
c	9	1	10	8
d	2	0	10	10
e	2	1	13	8
f	8	7	9	9
g	6	1	5	7
h	–	–	12	6
i	–	–	12	9
j	–	–	10	12
$\bar{x}$=	$6 \pm_4$	$3 \pm_3$	$10 \pm_3$	$8 \pm_2$

TABLE 6

DECREASED C2 SYNTHESIS BY BURN PT MØ

COLLECTED AT VARIOUS DAYS POST BURN

Plaques/ 10^6 MØ

After 4 Days Culture

PATIENT/CONT.	POST BURN DAYS 1 – 2		POST BURN DAYS 5 – 7	
	UNSTIM	STIM	UNSTIM	STIM
p1/c	7/10	32/27	0/8	5/22
p2/c	9/8	24/18	2/5	2/18
p3/c	2/10	12/20	0/10	6/18
p4/c	3/2	10/15	7/5	9/18

that there is a major and significant difference between MØ C synthesis by Group III burn patients and by normals (Table 6). After severe thermal injury, the MØ at the injury site should be activated to increase C synthesis. In fact, burn patient MØ are unable to respond to immune stimuli with increase MØ C synthesis. Consequently, our data imply that an immunoincompetent burn patient would not only have reduced C levels because of decreased lymphokine activity, but also that the MØ themselves would have reduced synthetic capacity. The level of fresh C available at the injury site for PMN chemotaxis and phagocytosis would be drastically reduced for these patients. We have only begun to monitor burn patients MØ complement synthesis.

In our C assays to date, we have only examined MØ C synthesis in already identified mitogen hyporesponsive burn patients (Group III). In addition, we have only examined the MØ C synthetic levels at 5-7 days post-injury. It is possible, therefore, that we are measuring the effects of T_s and/or increased PGE_2 rather than an inherent MØ dysfunction. Both T_s and PGE_2 could inhibit MØ C production. In the next few months we expect to monitor patient MØ C synthesis every 3 days from admission to demise in the same way we assess PA function. In these experiments we will determine if the defect in MØ C synthesis only occurs as a result of excessive regulatory cells. Additionally, we will examine patient T_s subsets for their ability to suppress normal individuals MØ C synthesis.

Severe thermal injury is known to augment the activity of at least one type of T_s. An $OKT8^+$ non-genetically restricted T_s has been identified by a number of investigators as appearing after severe thermal injury. The appearance of this expanded T_s activity has a negative prognosis for the patient. Several human T_s subsets have been identified. Any or all of these subsets may be expanded after thermal injury. We have already demonstrated that T_s can suppress MØ functions. We are in the process of defining what T_s subsets can suppress MØ function and developing assays to delineate the appearance of these excessive regulatory cells after severe burns. As illustrated in Table 7, we have shown that an $OKT8^+$ T_s will suppress MØ PA production. In addition, our data indicate that a T_s in the $OKT4^+$ population is also suppressive for MØ PA function (Table 8). MØ lysozyme production and TF activity are not depressed in MØ populations exposed to T_s. These data imply that T_s can specifically suppress fac MØ function (PA production) while leaving other MØ activities intact. In the coming year we will be employing these same techniques to delineate T_s subsets in the Group III burn patients.

TABLE 7. SUPPRESSION OF MØ PA PRODUCTION BY CONA ACTIVATED CELLS AND OKT8 DEPLETED CELLS

EXP. NO.	DONOR	CONTROL (PA CONTENT AS % FIBRINOLYSIS)	CONA CELLS	PERCENT SUPP.	OKT8 DEPLD	PERCENT SUPP.
321	SB	46.5	25.2	46%	35.1	25%
328	MM	33.3	24.7	26%	30.0	10%
387	BS	79.5	46.1	42%	60.1	25%
399A	RW	37.8	17.5	54%	23.7	27%
399B	RW	37.8	20.5	46%	32.9	13%
418	EH	49.8	30.2	39%	43.5	13%
423	CY	37.8	28.1	26%	42.2	-12%
430	TB	26.3	13.6	48%	17.5	33%
421	RW	38.5	28.6	26%	29.6	23%
422A	JM	49.7	37.9	24%	34.6	30%

TABLE 8. POSITIVELY SELECTED ConA STIMULATED CELLS RESULT IN SIGNIFICANT SUPPRESSION OF MØ PA PRODUCTION

EXP. NO.	DONOR	CONTROL (PA CONTENT AS % FIBRINOLYSIS)	ConA CELLS	% SUP.	T8(+)	% SUP.	T8(-)	% SUP
422	JM	49.7	37.9	24%	37.9	24%	ND	
428	BS	43.3	36.6	16%	31.9	26%	ND	
430	TB	26.3	13.3	48%	21.7	17%	20.2	23%
432	EH	62.3	^{D}58.4	6%	42.4	32%	49.6	20%
428A	BS	43.3	^{D}39.9	8%	31.9	26%	ND	

POSITIVELY SELECTED $ConA_6$ GENERATED CELLS WERE ADDED AT A CONCENTRATION OF 1×10^6 TO NORMAL HUMAN PERIPHERAL BLOOD MØ. THE SUPERSCRIPT $(D)_6$ DENOTES ConA CELLS ADDED AT A CONCENTRATION OF 1×10^6, RATHER THAN 5×10^6.

Decreased MØ function and augmented T_s and inh MØ activity seem to be the key defects in the immunoincompetent burn patient syndrome. Consequently, prophylactic therapy which is directed toward decreasing T_s, moderating PGE_2 activity, and/or increasing fac MØ function, should benefit burn victims. Utilizing our guinea pig (g.p.) burn model we have examined in vivo injection of TP5 (a thymopoetin pentapeptide) and indomethacin (a PGE_2 antagonist) for their modulation of the decreased antibody forming cell (AFC) response in our burned g.p. system. The experimental design was as follows: all male or all female, syngeneic age matched g.p. of either strain 2 or strain 13 were divided into 3 groups. One group was sham injured as previously described and served as controls for the AFC response. The second group was thermally injured and then their splenocytes were assayed in the AFC response. The third group was burned and initially injected with either 1 mg/kg/day of TP5 or 3 mg/kg/day 18 hours post-injury and then received 2 subsequent injections on each of the next 2 days. The three groups were assayed simultaneously. As can be seen in Table 9, the response of the burned g.p. group was markedly reduced from that of the sham injured control group simultaneously assayed. In contrast, the animals who received 1 mg/kg/day of TP5 for 3 days post-burn showed a significant increase ($p < .005$) in their AFC response over the burned group but still did not exhibit complete restoration to control response levels (Table 9).

In initial experiments, the difference between the 1 mg/kg dose and the 3 mg/kg dose were not significant. However, with further testing the 1 mg/kg dose appeared more effective p=.05. In another set of experiments, we examined in vivo administration of indomethacin as a prophylactic therapy. Indomethacin should prevent increased inh MØ synthesis of PGE_2 after burns. Again, the animals were divided into 3 groups, 2 burned and one control. One of the burn groups received indomethacin. The data (Table 10) supported the conclusion that indomethacin could partially restore the AFC response ($x = 40.5\% \pm 7.5$ of control). It is of particular interest that neither TP5 nor indomethacin by themselves could totally restore the AFC response after severe thermal insult. The therapeutic action of TP5 is directed at expanding the T_h population thereby moderating to some extent the depressive effect of T_s. In contrast, the action of indomethacin is to prevent excessive PGE_2 synthesis. In this case the target of the drug is presumably the inh MØ. Since neither treatment by itself was completely effective in restoring the AFC response, it suggests that at least two separate immune defects are generated by burns. In a preliminary set of experiments, we have examined the effect of combining both TP5 and indomethacin treatment in our burned g.p. model. As illustrated in Figure 3, combination of TP5

TABLE 9

EFFECT OF IN VIVO ADMINISTRATION OF TP5 ON BURN INDUCED SUPPRESSION OF AFC RESPONSE

% SHAM INJURED CONTROL GENERATED AFC PER 10^6 RECOVERED CELLS

	I	II	III	IV	V	VI	VII
1 MG/KG INJECTED X 3							
BURN	1%	1%	17%	1%	10%	5%	
BURN & INJ TP5	35%	13%	68%	30%	24%	48%	
3 MG/KG INJECTED X 3							
BURN	4%	1%	1%	1%	7%	10%	6%
BURN AND INJ TP5	46%	43%	17%	28%	49%	25%	31%

T A B L E 10

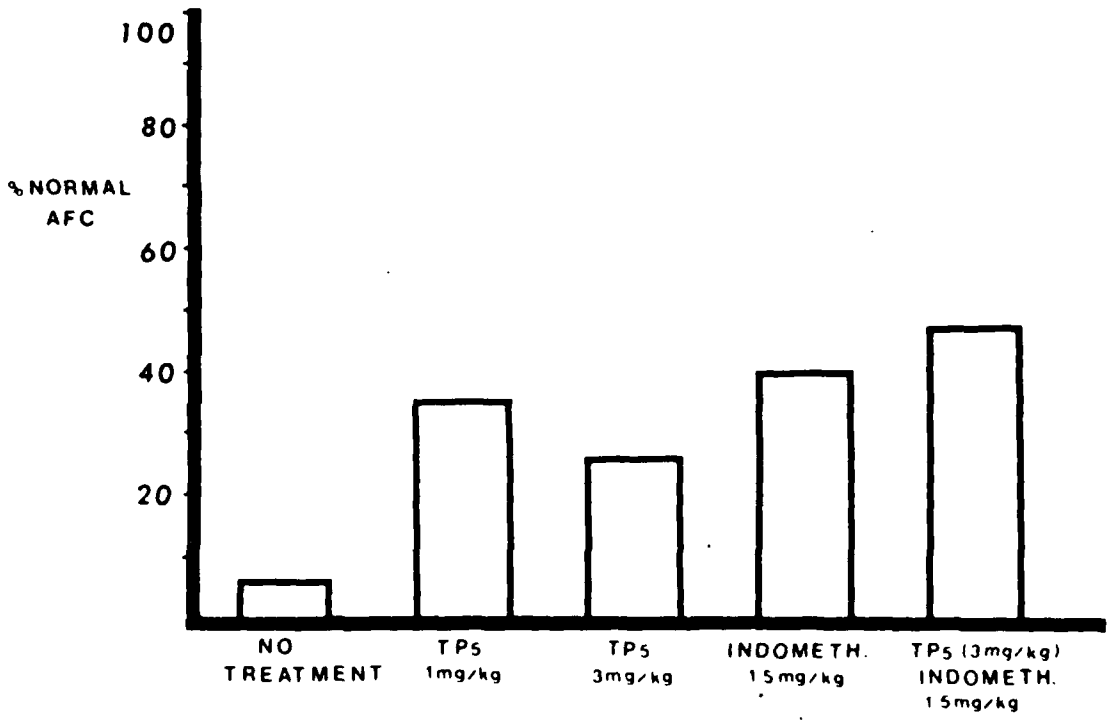
Effect of In Vivo Administration of Indomethacin in
Burn Induced Suppression of AFC Response

1.5 mg/kg Indomethacin Injected 3x Intraperitoneally Post-Burn
% of control

Burn	6	1	8	3	11	4
Indomethacin	44	35	50	38	45	31

FIGURE 3

EFFECT OF TP5 AND INDOMETHACIN ON BURNS



and indomethacin appeared to be slightly more effective in restoring immune function than either drug by itself. In our present studies, we are examining this combinational therapy and using various different levels of TP5 and indomethacin. It is possible that lower doses of both drugs would be more effective in combination, than the higher levels we employed when testing each drug alone.

In another set of experiments we attempted to augment fac MØ function by injecting dextran. The results from these experiments were inconclusive. Multiple injection of dextran (i.e., 3 cc 3 times a day for 3 days) resulted in depressed AFC responses in normal control animals receiving dextran. These animals splenocytes were extremely difficult to tease into single cell suspensions. The cells tended to clump together. In addition, the injection procedure itself was traumatic for the animals and tended to produce necrosis at the injection site as well as thrombosis in the repeatedly injected veins. Consequently, we have discontinued these experiments until we have perfected a means for continuous infusion of the g.p. We are examining both installation of a subclavian catheter (a method in use in Dr. George Sheldon's laboratory) and placement of a venous line under anesthesia for infusing all our drugs. Once an infusion model has been perfected, we will reassess all of the drugs currently under study (Indo, TP5, and Dextran) as well as any new prophylactic modalities that may be suggested by our experiments.

This contract period has been particularly productive. We have developed the MØ C assay, and have accumulated data indicating that this new assay is monitoring an important burn altered MØ function. We are improving and refining the PGE_2 assay while gathering important new indications of the inimical effects of post-burn elevation of MØ PGE_2 on immune function. We also now have the capacity to identify human T_s subsets using the fluorescence cell sorter and expect to expand this capacity in the coming year. Most important, we are now beginning to examine various prophylactic treatments for their efficacy in reversing a number of burn mediated immune defects. We expect even more interesting data and several publications to result from experiments now in progress.

REFERENCES

1. Alpert SE, Auerbach HS, Cole FS, Colten HR: 1983. Macrophage maturation: Differences in complement secretion by marrow, monocytes, and tissue macrophages detected with an improved hemolytic plaque assay. J of Immunol 130(1):102.

2. Wahl LM: 1981. Production and quantitation of prostaglandins. In Manual of Macrophage Methodology: Collection, Characterization and Function (Herbert B. Herscowitz, *editor*). Published by M. Dekker, New York.

3. Beer DJ, Dinarello CA, Rosenwasser LJ, Rocklin RE: 1982. Human monocyte-derived soluble product(s) has an accessory function in the generation of histamine- and concanavalin A-induced suppressor T cells. J Clin Invest 70:393.

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